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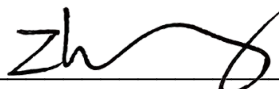
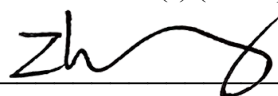
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FOR THE DEGREE OF

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IN

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Identification of Messenger Molecule Between Mammalian and Bacterial Cells

By

Anna Fraser-Philbin and Alexander Heiler

SENIOR DESIGN PROJECT REPORT

Submitted to

the Department of Bioengineering

of

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Identification of Messenger Molecule Between Mammalian and Bacterial Cells

Anna Fraser-Philbin, Alex Heiler

Department of Bioengineering

Santa Clara University

2020

ABSTRACT

The rapid escalation of the antibiotic resistance crisis has brought attention to the decline in efficiency of antibiotics which have long been a cornerstone of modern medicine. This project aims to provide novel drug targets for the creation of anti-infective immunotherapies that can treat drug-resistant infections. The identification of said drug target(s) (Molecule X) will allow for the development of an antibody based drug that will neutralize bacterial virulence rather than killing the bacteria. Molecule X will be bound using the protein Sortase A (SrtA). SrtA is a surface protein that controls the virulence of gram positive bacteria by anchoring virulence factors to the cell surface. Molecule X is the signal from mammalian cells that initiates this process. Blocking this signal prevents the activation of bacterial virulence.

SrtA is obtained from *E. coli* cells using affinity chromatography and Molecule X is obtained by collecting the entire mammalian proteome of Chinese Hamster Ovary (CHO) cells. The SrtA and mammalian proteome are mixed together with a crosslinker to covalently bind Molecule X to SrtA. The cross-linked products are purified and analyzed using liquid chromatography with tandem mass spectrometry (LC-MS-MS) and the proteins are identified using a proteomic database. The resulting data is analyzed for Molecule X candidates by grouping the proteins into clusters based on function and subcellular location. The identified clusters are calcium ion dependent, membrane associated, G protein-coupled receptors (GPCR) associated, and adenosine triphosphate (ATP) or guanosine triphosphate (GTP) binding proteins.

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TABLE OF CONTENTS

ABSTRACT	iii
ACKNOWLEDGEMENTS	iv
LIST OF FIGURES	ix
LIST OF TABLES	x
LIST OF ABBREVIATIONS	xi
CHAPTER 1: INTRODUCTION	1
1.1 BACKGROUND	1
1.1.1 ANTIBIOTIC RESISTANCE CRISIS	1
1.1.2 STAPHYLOCOCCUS AUREUS INFECTIONS	2
1.1.3 VIRULENCY THROUGH SORTASE A	3
1.2 REVIEW OF FIELD.LITERATURE	4
1.3 CRITICS OF CURRENT LITERATURES	5
1.4 PROJECT GOAL	6
1.5 BACKUP PLAN	6
1.6 SIGNIFICANCE	6
1.7 TEAM AND MANAGEMENT	7
1.8 BUDGET	7
1.9 TIMELINE	7
CHAPTER 2: CULTURES AND INDUCTION	9
2.1 INTRODUCTION	9
2.2 KEY CONSTRAINTS	9
2.3 DESIGN DESCRIPTION AND SOLUTIONS TO PROBLEM	9
2.4 DETAILED SUPPORTING ANALYSIS	9
2.5 EXPECTED RESULTS	9
2.6 BACKUP PLAN	10
2.7 MATERIALS AND METHODS	10
2.7.1 PLATING TRANSFORMED CELLS	10
2.7.2 CULTURING E. COLI CELLS AND INDUCING PROTEIN EXPRESSION	10
2.8 RESULTS	11
2.9 DISCUSSION	
CHAPTER 3: PURIFICATION OF SORTASE A	12
3.1 INTRODUCTION	12
3.2 KEY CONSTRAINTS	12

3.3 DESIGN DESCRIPTION AND SOLUTIONS TO PROBLEM	12
3.4 DETAILED SUPPORTING ANALYSIS	12
3.5 EXPECTED RESULTS	13
3.6 BACKUP PLAN	13
3.7 MATERIALS AND METHODS	13
3.7.1 LYSING E. COLI CELLS	13
3.7.2 PREPARATION OF NI-NTA BEADS	13
3.7.3 PURIFICATION OF SORTASE A	14
3.7.4 RUNNING SDS-PAGE GEL	14
3.7.5 COOMASSIE STAINING	15
3.8 RESULTS	15
3.9 DISCUSSION	16
 CHAPTER 4: EXTRACTION OF MAMMALIAN PROTEOME	 17
4.1 INTRODUCTION	17
4.2 KEY CONSTRAINTS	17
4.3 DESIGN DESCRIPTION AND SOLUTIONS TO PROBLEM	17
4.4 DETAILED SUPPORTING ANALYSIS	18
4.5 EXPECTED RESULTS	18
4.6 BACKUP PLAN	18
4.7 MATERIALS AND METHODS	19
4.7.1 EXTRACTION OF CHO CELL PROTEOME	19
4.8 RESULTS	19
4.9 DISCUSSION	19
 CHAPTER 5: CROSS-LINKING	 20
5.1 INTRODUCTION	20
5.2 KEY CONSTRAINTS	20
5.3 DESIGN DESCRIPTION AND SOLUTIONS TO PROBLEM	20
5.4 DETAILED SUPPORTING ANALYSIS	21
5.5 EXPECTED RESULTS	22
5.6 BACKUP PLAN	22
5.7 MATERIALS AND METHODS	22
5.7.1 EDC CROSS-LINKING	22
5.7.2 BPM CROSS-LINKING	22
5.7.3 PURIFICATION OF CROSS-LINKED PRODUCTS	23
5.7.4 RUNNING SDS-PAGE GEL	23
5.7.5 SILVER STAINING	24
5.8 RESULTS	24

5.9 DISCUSSION	24
CHAPTER 6: DIGESTION AND MASS SPECTROMETRY ANALYSIS	25
6.1 INTRODUCTION	25
6.2 KEY CONSTRAINTS	25
6.3 DESIGN DESCRIPTION AND SOLUTIONS TO PROBLEM	25
6.4 DETAILED SUPPORTING ANALYSIS	25
6.5 EXPECTED RESULTS	25
6.6 BACKUP PLAN	26
6.7 MATERIALS AND METHODS	26
6.7.1 CHEMICALS AND INSTRUMENTATIONS	26
6.7.2 SAMPLE PREPARATION	26
6.7.3 NANOLC	27
6.7.4 MASS SPECTROMETRY	27
6.7.5 DATA ANALYSIS	27
6.8 RESULTS	28
6.9 DISCUSSION	28
CHAPTER 7: SUMMARY AND CONCLUSION	29
7.1 PROJECT SUMMARY	29
7.2 EVALUATION OF MILESTONES	29
7.3 FUTURE WORK	29
CHAPTER 8: ENGINEERING STANDARDS AND REALISTIC CONSTRAINTS	31
8.1 ECONOMIC	31
8.1.1 COST OF DEVELOPMENT	31
8.1.2 COST OF OWNERSHIP	32
8.1.3 USER’S PERSPECTIVE	32
8.1.4 IS IT WORTH IT?	32
8.2 SUSTAINABILITY	32
8.2.1 WHY SUSTAINABILITY?	32
8.2.2 MATERIAL RESOURCES	33
8.2.3 ENERGY RESOURCES	33
8.2.4 SUSTAINABLE ENGINEERING	33
8.3 ETHICS	34
8.3.1 ETHICAL APPROACHES	34
8.3.2 FRAMEWORK FOR THINKING ETHICALLY	34
8.3.3 ETHICAL CONSIDERATIONS	34
8.4 HEALTH AND SAFETY	35

8.4.1 ENGINEERING FAILURES	35
8.4.2 ENGINEERING FOR A HEALTHIER AND SAFER WORLD	35
8.4.3 UNANTICIPATED CONSEQUENCES	36
8.4.4 IN DEFENSE OF FAILURE	36
8.4.5 RISKS	37
8.5 SOCIAL	37
8.5.1 ROLE OF TECHNOLOGY IN SOCIETY	37
8.5.2 DECIDING WHAT IS TO BE DONE	37
8.5.3 THE ROLE OF THE ENGINEER	38
8.5.4 HUMAN NEEDS	38
BIBLIOGRAPHY	39
APPENDICES	
APPENDIX A: TABLES	A-1
APPENDIX B: ADDITIONAL CROSSLINKER GELS	B-1
APPENDIX C: LC-MS-MS DATA	C-1

List of Figures

Figure 1. Worldwide prevalence of MRSA	CH 1
Figure 2. Covalent attachment of proteins to the peptidoglycan by <i>S. aureus</i> SrtA	CH 1
Figure 3. Project tasks	CH 1
Figure 4. SDS-PAGE gel of SrtA purification	CH 3
Figure 5. Average concentration of protein for each purification step	CH 3
Figure 6. Homogenizer used for mammalian proteome extraction	CH 4
Figure 7. Crosslinker mechanisms of a) EDC and b) BPM	CH 5
Figure 8. SDS-PAGE gels of a) initial EDC cross-linking, b) initial BPM cross-linking, and c) optimized BPM cross-linking	CH 5
Figure 9. Breakdown of discovery and development costs from the 2010 Eli Lilly study	CH 8
Figure 10. SDS-PAGE gels of BPM cross-linking on a) 2/25 and b) 3/6	APPX B
Figure 11. Mass spectrometry results from MtoZ Biolabs	APPX C

List of Tables

Table 1. <i>E. coli</i> cell pellet masses after culturing and protein induction	APPX A
Table 2. Protein concentrations for each SrtA purification step	APPX A
Table 3. Proposed Project Budget	APPX A
Table 4. Project Budget	APPX A

List of Abbreviations

Acetonitrile	ACN
Adenosine triphosphate	ATP
Benzophenone maleimide, 4-(N-maleimido) benzophenone	BPM
Chinese Hamster Ovary	CHO
DL-dithiothreitol	DTT
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide	EDC
Formic acid	FA
G protein-coupled receptor	GPCR
Guanosine triphosphate	GTP
Iodoacetamide	IAA
Luria-Bertani	LB
Methicillin-resistant <i>S. aureus</i>	MRSA
Monoclonal antibody	mAb
Mortality factor 4-like protein 2	MORF4L2
N-hydroxysulfosuccinimide sodium salt	Sulfo-NHS
Pentatricopeptide repeat-containing protein 1	PTCD1
Sortase A	SrtA
Transfer RNA	tRNA
tRNA-derived small RNA fragments	tRFs

CHAPTER 1: INTRODUCTION

1.1 Background

1.1.1 Antibiotic Resistance Crisis

Bacterial infections are mostly treated with antibiotics, which, through various mechanisms, kill the infectious bacteria. Common antibiotics are various types of penicillin, including methicillin, oxacillin, flucloxacillin, and dicloxacillin. Due to the widespread use of penicillin in the 1940s and methicillin in the 1960s, bacteria have mutated to resist the antibiotics¹. Importantly, methicillin resistance provides resistance to most β -lactam antibiotics that are used to treat infection.

One significant strain of antibiotic resistant bacteria is methicillin-resistant *S. aureus* (MRSA). Initially, it was only found in patients who received healthcare, as the bacteria in the hospitals were exposed and desensitized to methicillin. However, the significance of MRSA escalated as it was later detected in individuals who had not come in contact with healthcare. Alarming, methicillin resistance spread, as, by the 1980s, the first cases of MRSA were found in indigenous populations in Australia, who were isolated from modern medicine. By the 1990s children and healthy adults were found to carry MRSA, and by the mid-2000s it was also detected in livestock. Resistance spread between different strains of *S. aureus*, as strains that were never exposed to methicillin began to gain resistance across the globe (Fig. 1). This rise in resistance led to the search for ways to treat bacterial infections without killing the bacteria. By treating infections in a nonlethal manner, the bacteria will face less evolutionary pressure to mutate and gain resistance against the anti-infective treatment.

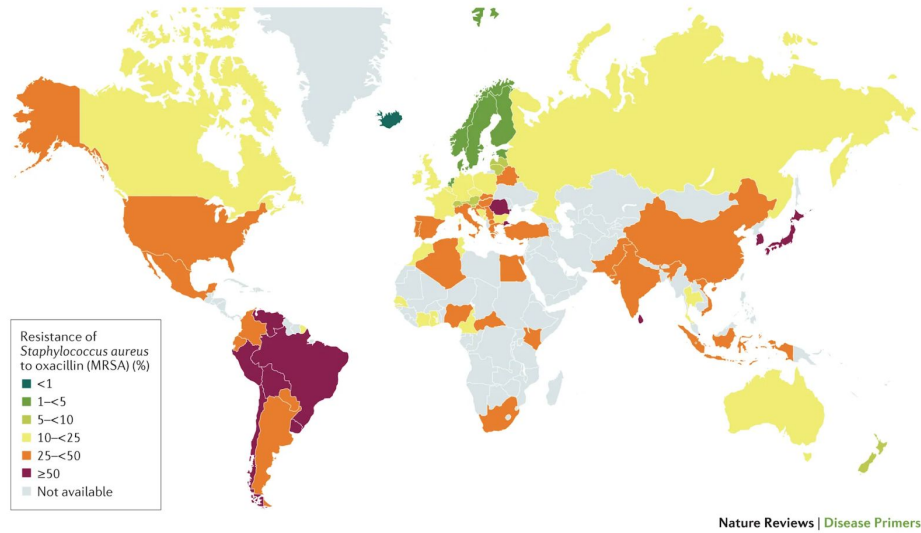


Figure 1. Worldwide prevalence of MRSA. This map shows the global distribution of MRSA that is resistant to oxacillin¹.

1.1.2 *Staphylococcus aureus* Infections

Bacterial infection is a common disease prevalent in all parts of the world. While bacterial species of both the gram positive and gram negative variety are pathogenic, gram positive bacteria are especially threatening due to protective peptidoglycan layer. Specifically, the gram positive bacterium *Staphylococcus aureus* is a major public health concern and is the most widely researched and understood bacterium. Able to infect any tissue, *S. aureus* causes a range of symptoms with varying severity, including skin and soft-tissue infections, septicemia, and pneumonia.

While everyone is susceptible to infection, the most vulnerable populations are the young, the elderly, and the immuno-suppressed. Especially in low-income countries, *S. aureus* infection rates are highest in children under one year of age with mortality rates estimated at 50%. While infection is less pervasive in developed countries, it still causes approximately 300,000 hospitalizations per year². Because *S. aureus* infection is such a global and universal hazard, preventing infection provides opportunity to greatly affect the overall health of the global community.

1.1.3 Virulence through Sortase A

Sortases are proteins commonly found on gram positive bacteria. Sortase A (SrtA) is a type A housekeeping transpeptidase that anchors surface proteins by covalently binding their carboxyl terminus ends³. Initially discovered in *S. aureus*, homologs of SrtA have been found in most gram positive bacteria. The wide variety of surface proteins that SrtA and its variants bind mean that mutants of the protein will often affect the virulence of the bacteria. Proteins that are anchored by SrtA can be identified by the presence of a conserved C-terminal domain motif, the amino acids LPXTG, which acts as the sortase recognition sequence⁴. The currently accepted anchoring mechanism involves the SrtA cleaving the surface protein at the motif to remove the C-terminal hydrophobic region (Fig. 2).

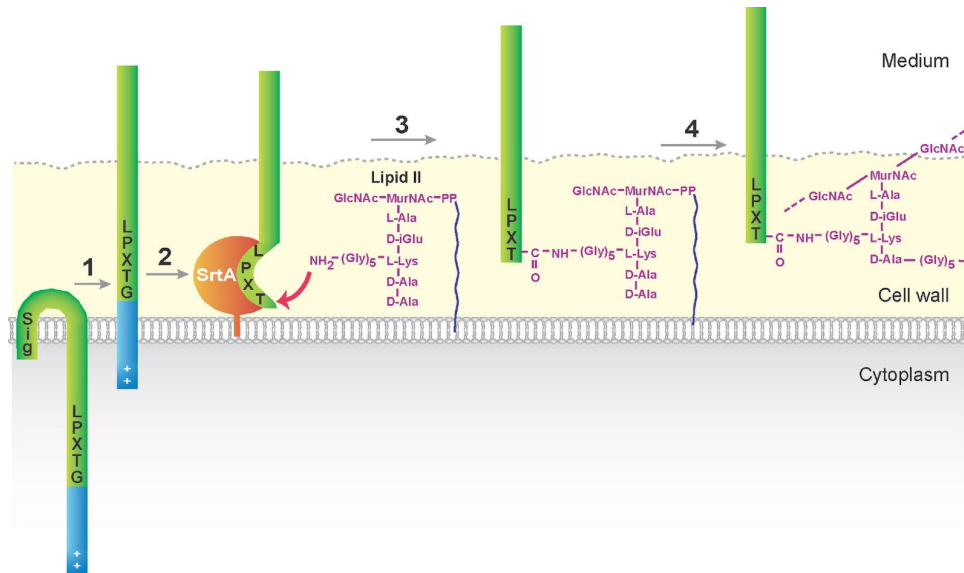


Figure 2. Covalent attachment of proteins to the peptidoglycan by *S. aureus* SrtA. (Step 1) Surface proteins are first secreted through the membrane and the N-terminal signal sequence (Sig) is removed. The C-terminal hydrophobic domain and positively charged tail (blue) retain the protein in the membrane and allow the LPXTG motif to be recognized by the membrane-bound sortase enzyme (orange). (Step 2) Sortase then cleaves the LPXTG motif between the threonine and glycine residues (95) by a reverse-protonation catalytic mechanism, which releases the C-terminal hydrophobic region and charged tail. The N-terminal portion of the cleaved protein forms an acyl-enzyme intermediate with sortase. (Step 3) This is resolved following a nucleophilic attack (red arrow) by an amine group on the cell wall (cw) precursor, lipid II. (Step 4) The protein-linked lipid II molecule is then

incorporated into the mature cw by the transglycosylation and transpeptidation reactions of bacterial peptidoglycan synthesis³.

In addition to attaching cell surface proteins, SrtA contributes to the pathogenesis of *S. aureus*. Researchers infected mice with both wildtype and non-SrtA expressing *S. aureus*. While the wildtype bacteria formed abscesses in the mice kidneys, the mutated bacteria exhibited reduced virulence and ability to form kidney abscesses^{5,6}. Furthermore, virulence factors surfaces have been shown to be controlled by sortase enzymes, specifically SrtA⁷. These virulence factors control adhesion, host-cell damaging, and immunomodulatory molecule production¹. Therefore, SrtA directly promotes bacterial infection in host tissue and is a promising therapeutic target to prevent bacterial infections⁸.

Specific conditions must be met in order to ensure that SrtA retains its native active structure in vitro. Beyond standard protein buffer conditions, research suggests that SrtA also requires the presence of the metal ion activator Ca^{2+} . The presence of Ca^{2+} helps to stabilize a highly dynamic β loop that normally blocks off the binding site in SrtA⁹.

1.2 Review of Field/Literature

Currently, there are limited alternatives to antibiotics to treat bacterial infections. Two fields that are working to develop alternatives are biomaterials and vaccines. The area of nanotechnology is especially promising thanks to the natural antimicrobial properties of silver nanoparticles¹⁰. One application is coating biomedical devices and implants with the nanoparticles to ensure the product integrates safely with the patient¹¹. Another application is the use of topical nanoparticle creams and ointments that have been shown to be effective in fighting a number of bacterial infections and for wound healing¹². While the applications are diverse, how the nanoparticles interact with and disrupt the bacteria is poorly understood. Another strategy against infection is an anti-infective immunotherapeutic. This proposed vaccine would boost the immune response of the patient against bacteria. However, clinical trials show the vaccine has little effect on infection prevention. Research has shown patients who recover from infection gain no additional immunity against subsequent infection. While different approaches and more

complex vaccines are being explored, more progress must be achieved before developing an effective vaccine¹³.

Researchers have previously discovered a number of types of SrtA inhibitors, though none have proven efficient nor clean enough to develop for therapeutic use as of yet. These inhibitors can be sorted into the classes of non-specific, peptide-analogues, natural products, and synthetic small molecules. Non-specific inhibitors work by modifying a side chain involved in the transpeptidation, but they are not specific to sortases and have low potency.

Peptide-analogues work by exhibiting the LPXTG motif, but they too have only moderate efficiency. Natural product inhibitors have been gaining interest in the past couple of decades and many have shown promise in inhibiting both SrtA and SrtB *in vitro* while not affecting bacterial viability. Few synthetic small molecule inhibitors have been developed, but they too show promise due to high efficacy and limited effect on bacterial cell growth¹⁴.

The signal molecule between SrtA and mammalian cells was hypothesized to be a membrane-associated protein. An extracellular protein could easily be sensed and bound by SrtA, allowing the bacteria to induce virulence and infect the mammalian cell. However, recent research on communication between nitrogen-fixing Rhizobia bacteria and plant roots shows transfer RNA (tRNA)-derived small RNA fragments (tRFs) are the messenger molecule between the two cells¹⁵. tRFs are synthesized in the cytosol of the Rhizobia, so the signal must be excreted before binding to the receptors on the plant cells to induce root nodulation (Ren et. al). Because the signal between bacteria and plant cells was shown to be intracellular, the search for the signal between bacterial and mammalian cells was expanded from only membrane proteins to the entire mammalian proteome.

1.3 Critics of Current Literatures

Because bacteria are resisting the main way to treat them, antibiotics, there is active research to find an alternative. Infection is an interaction between the pathogen and the host. The two main ways to alter and disrupt this relationship are to target the pathogen or the host. This project focuses on a novel approach to target the communication between the two. This approach follows the strategy to prevent the infection without actually killing the pathogen. The lethality

causes the bacteria to mutate and develop resistance, so stopping the infection will prevent any harm from the infection without providing the selective pressure to acquire resistance to the treatment. The bacteria should not develop resistance since blocking the signaling mechanism does not kill nor directly affect the bacteria. This drug would be in a class of anti-infectives. To achieve this, there needs to be more understanding of how the pathogen and the host communicate and the pathways that could be disrupted or altered in the presence of the anti-infective.

1.4 Project Goal

This project aims to further the search for successful alternatives to antibiotics by identifying a novel drug target to help develop an anti-infective immunotherapy. This molecule should allow for the development of an antibody based drug that will neutralize bacterial virulence instead of resulting in the death of the bacteria. To accomplish this, the protein SrtA is used as the bacterial receptor with which to capture the potential drug target.

1.5 Backup Plan

The backup plan is to use different chemical crosslinkers. Since different crosslinkers react via different mechanisms, another crosslinker may be more suitable to capture Molecule X with SrtA. Two backup crosslinkers are dibromobimane and 4-benzoylbenzoic acid, succinimidyl ester.

1.6 Significance

By searching for the messenger molecule between bacterial and mammalian cells, this project explores a novel approach to treating infections. Targeting the communication between the two cell types provides opportunity to stop the infection without killing the bacteria. This solution is especially important for populations particularly vulnerable to antibiotic-resistant bacteria, including the immunocompromised, patients in hospitals, and people residing in low-income regions of the world.

Besides potentially treating a resistant disease, an anti-infective immunotherapeutic would have a major economic impact. In the US, the cost of treating an antibiotic resistant infection per patient is up to \$29,069, with the annual national cost totalling roughly \$20 billion¹⁶.

In addition to providing an alternative to antibiotics, which is greatly needed in the worldwide crisis of antibiotic-resistant superbugs, this approach can be translated to other types of infections and diseases.

1.7 Team and Management

As a two person team, both researchers were responsible for understanding and performing all technical procedures for the project. The administrative duties were split up evenly. Mr. Heiler was the main contact for the School of Engineering and ordering materials. Ms. Fraser-Philbin was the main contact for their advisor and scheduling meetings. The workload for deliverables was equally shared and completed together. All other duties were divided equally.

1.8 Budget

The Undergraduate Programs Senior Design Grants awarded provided \$1000 towards this project, while Xilinx granted \$240 towards expanding the scope of the project. The grant money was used towards purchasing all materials and reagents necessary to identify Molecule X (see Appendix A).

1.9 Timeline

To complete this project, the overall tasks included literature review, experimental trials, and thesis development (Fig. 3). The overall processes were conducted in parallel. The literature review informed our experimental design, and the results from each experiment were compiled and included in the thesis.

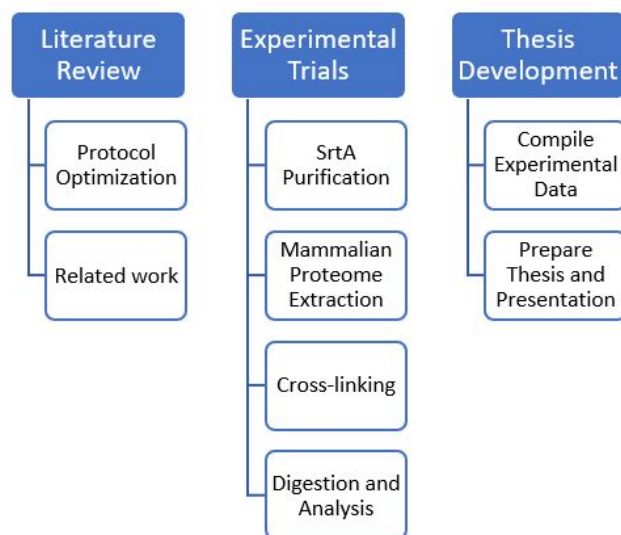


Figure 3. Project tasks. Each column was worked on simultaneously, while the tasks in each row were completed from top to bottom.

CHAPTER 2: CULTURE AND INDUCTION

2.1 Introduction

This experiment worked to obtain a large quantity of bacterial cells expressing high levels of the desired protein, SrtA. A bacterial stab of transformed *E. coli* cells, purchased from Addgene, was first plated, then cultured before protein expression was induced.

2.2 Key Constraints

The plasmid the bacterial cells were transformed with have the genes for both the modified SrtA and kanamycin resistance. The agar plates and LB (Luria-Bertani) broth have kanamycin added to select for transformed bacteria.

Another constraint was the long culture times necessary for each stage, moving from the agar plates to small culture inoculations, to big culture inoculations, then to protein induction.

2.3 Design Description and Solutions to Problem

Initial trials of culturing and inducing the bacteria resulted in very low yields of SrtA. The protocol was optimized to increase protein yield. Therefore, the induction time was reduced from 18 hrs to between 4 and 6 hrs.

2.4 Detailed Supporting Analysis

After initial trials, literature review of protein induction protocols showed shorter induction times, usually between 4 and 6 hrs, were more successful.

2.5 Expected Results

The final big culture inoculation was expected to produce a cell pellet weighing about 10 g. Following purification, a cell pellet of this size should yield around 24 mg of SrtA. The bacterial cells were expected to have low mutation rates and plasmid loss.

2.6 Backup Plan

Before centrifuging the induced large culture, 5 mL of cell culture broth was collected and used to make a DMSO stock of transformed cells and stored in the -80 °C freezer. For subsequent experiments, a sliver of the stock was resuspended in LB broth and cultured, eliminating the need for plating.

2.7 Materials and Methods

2.7.1 Plating Transformed Cells

50 µL of transformed cells were spread on an LB + Agar plate in a zigzag line. The colonies were grown upside down in a 37 °C incubator overnight and stored in a 4 °C refrigerator.

2.7.2 Culturing *E. coli* Cells and Inducing Protein Expression

37.5 g of LB powder was mixed into 1500 mL DI water in a 2L Erlenmeyer flask and autoclaved for 1 hour. After cooling, 75 mg of 50 µg/mL kanamycin sulfate was added to the LB media. 5 mL of LB media was pipetted into 2 small culture tubes and one *E. coli* colony was added to each tube. The small cultures were inoculated in an incubator at 37 °C and 225 rpm until reaching an OD₆₀₀ of 0.6. The small cultures were poured into the Erlenmeyer flask and incubated at 37 °C and 225 rpm until reaching an OD₆₀₀ of 0.6. Protein expression was induced by adding 357.45 mg IPTG to the flask and incubated at 37 °C and 225 rpm for 4-6 hours. The cell solution was distributed into 4 500mL centrifuge bottles and centrifuged for 30 minutes in a J-10 Centrifuge Rotor at 4 °C and 10,000 rpm. The supernatant was discarded and the cell pellets were stored in a -80 °C freezer.

2.8 Results

After plating, small culture inoculation, and big culture inoculation, the bacterial cells were successfully collected through centrifugation. For each trial, cell pellets averaged 9.3 g for 1.5 L large culture (see Appendix A).

2.9 Discussion

This protocol consistently produced a desirable amount of *E. coli* within the described timeframe. Later experiments confirmed that the *E. coli* expressed SrtA protein.

CHAPTER 3: PURIFICATION OF SORTASE A

3.1 Introduction

This experiment extracts and purifies the protein SrtA from the cultured *E. coli* cells with high efficiency and yield. The same finalized protocol is also used to purify the cross-linked products.

3.2 Key Constraints

Because the SrtA was utilized in subsequent experiments to capture Molecule X, it had to be fully functional and structurally intact. Therefore, the purification was performed under native conditions to prevent protein denaturation. The pH needed to be slightly basic, and calcium was added to the buffers, which was incompatible with previously used PBS buffers. More washing steps was required because the buffers were less harsh and less effective. Also, because the Molecule X-SrtA complex would later be analyzed using LC-MS-MS, all the reagents had to be mass spectrometry compatible.

3.3 Design Description and Solutions to Problem

The protocol was optimized to increase capture and elution of SrtA as well as reduce binding of non-specific products. A bead buffer was added to better prepare the beads for binding to the 6x histidine tag on SrtA. All the buffers used Tris-HCl instead of phosphate to be compatible with the CaCl_2 . Also, 2-mercaptoethanol, glycerol, and increased NaCl were added to decrease non-specific binding and increase protein stability. Furthermore, the number of washing steps and RPM of centrifugation were increased to more effectively wash away non-specific products.

3.4 Detailed Supporting Analysis

An article depicting the structure of Srt A from Clancy et al. suggested calcium ions are required for the proper function of Srt A⁹. Therefore, to best emulate native conditions, CaCl_2 was included in the purification buffers. Also, a Qiagen manual describing Ni-NTA affinity

chromatography purification described how the addition of 2-mercaptoethanol and glycerol improved purification efficiency.

3.5 Expected Results

Following purification, the eluted solution was expected to contain a high yield of pure SrtA. An SDS-PAGE gel was used to confirm both the presence and purity of the SrtA, which should appear as a single dark band around 21 kDa. A nanodrop spectrophotometer was used to measure the protein concentration of the elution.

3.6 Backup Plan

If the eluted SrtA did not reach a purity required to proceed to later experiments, both FPLC and ion exchange chromatography would be used to further purify the SrtA. Also, the recombinant SrtA used has the 6x histidine tag on the N-terminus. If the N-terminal tagged SrtA was not successfully purified, then C-terminal tagged SrtA would be used.

3.7 Materials and Methods

3.7.1 Lysing *E. coli* cells

The cell pellet was thawed on ice for 20 minutes. 10 mg of lysozyme from Fisher Scientific was added to 10 mL of lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 0.5 mM CaCl₂, 5 mM 2-mercaptoethanol, 10 mM imidazole, and 0.09 mM PMSF, pH 8). The lysis solution was used to resuspend the cell pellet and rocked for 1 hour at 4 °C. The cell solution was transferred to a pointed tube and sonicated on ice, with 4 second pulse on and 59 second pulse off for 1.5 hours total. The sonicated solution was centrifuged using a J-17 Centrifuge Rotor at 4 °C and 10,000 rpm for 1 hour.

3.7.2 Preparation of Ni-NTA Beads

2 mL of Qiagen Ni-NTA resin bead slurry was used for every 1 L of cell culture. 5 mL of bead buffer (50 mM Tris-HCl, 200 mM NaCl, 0.5 mM CaCl₂, 5 mM 2-mercaptoethanol, and 0.09 mM PMSF, pH 7.4) was added to the bead slurry and centrifuged at 4 °C and 2000 rpm for

5 minutes. The bead buffer was discarded without disrupting the beads, and 5 mL of fresh bead buffer was added to the bead solution. The solution rocked for 30 minutes at 4 °C and was centrifuged at 4 °C and 2000 rpm for 5 minutes. The bead buffer was discarded and 5 mL of lysis buffer was added to the beads. The solution was centrifuged at 4 °C and 2000 rpm for 5 minutes. The supernatant was discarded.

3.7.3 Purification of Sortase A

The supernatant from the cell lysis solution was added to the clean Ni-NTA beads and rocked at 4 °C for 2 hours. The binding mixture was centrifuged at 4 °C and 2500 rpm for 1 hour. The flow through supernatant was collected and 5 mL of wash buffer (50 mM Tris-HCl, 700 mM NaCl, 0.5 mM CaCl₂, 5 mM 2-mercaptoethanol, 20 mM imidazole, and 0.09 mM PMSF, pH 8) was added to the bead solution. The solution was rocked at 4 °C for 20 minutes then centrifuged at 4 °C and 2000 rpm for 5 minutes. The wash supernatant was collected, and the bead solution was washed 4 more times. The beads were eluted with elution buffer (50 mM Tris-HCl, 200 mM NaCl, 0.5 mM CaCl₂, 5 mM 2-mercaptoethanol, 250 mM imidazole, and 0.09 mM PMSF, pH 8). First 2 mL of the elution buffer was added and the solution rocked at 4 °C for 20 minutes. The solution was centrifuged at 4 °C and 2000 rpm for 5 minutes and the first elution supernatant was collected. Then 1.5 mL of the elution buffer was added and the solution rocked at 4 °C for 20 minutes. The solution was centrifuged at 4 °C and 2000 rpm for 5 minutes and the second elution supernatant was collected. The flow through, wash supernatants, and elution supernatants were stored at -20 °C.

3.7.4 Running SDS-PAGE Gel

For the flow through, the 5 washes, and the 2 elutions, 48 µL of each sample was mixed with 12 µL of 5X loading dye in an Eppendorf tube. A gel from GenScript was attached to the Invitrogen SDS-PAGE apparatus and the container was filled with GenScript running buffer. 10 µL of Thermofisher 26628 protein ladder and each sample was loaded into the gel and ran at 100 V for 1.5 hours.

3.7.5 Coomassie Staining

The gel was washed with DI water then immersed in coomassie blue dye (50% methanol, 10% acetic acid, 40% DI water, 0.25% (w/v) coomassie blue) for 45 minutes. The gel was washed with DI water then immersed in a destaining mixture (50% DI water, 40% methanol, 10% acetic acid) for 24-48 hours. The gels were imaged with a LAS 500 Imager and stored at 4 °C.

3.8 Results

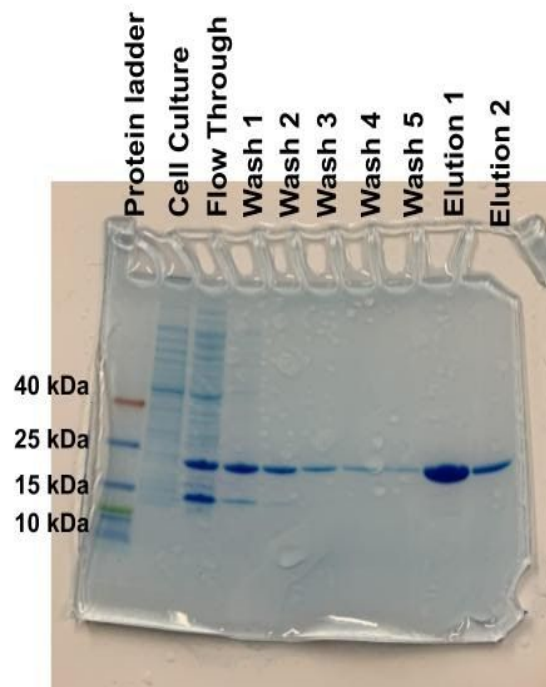


Figure 4. SDS-PAGE gel of SrtA purification. The intense dark bands below 25 kDa correspond with SrtA.

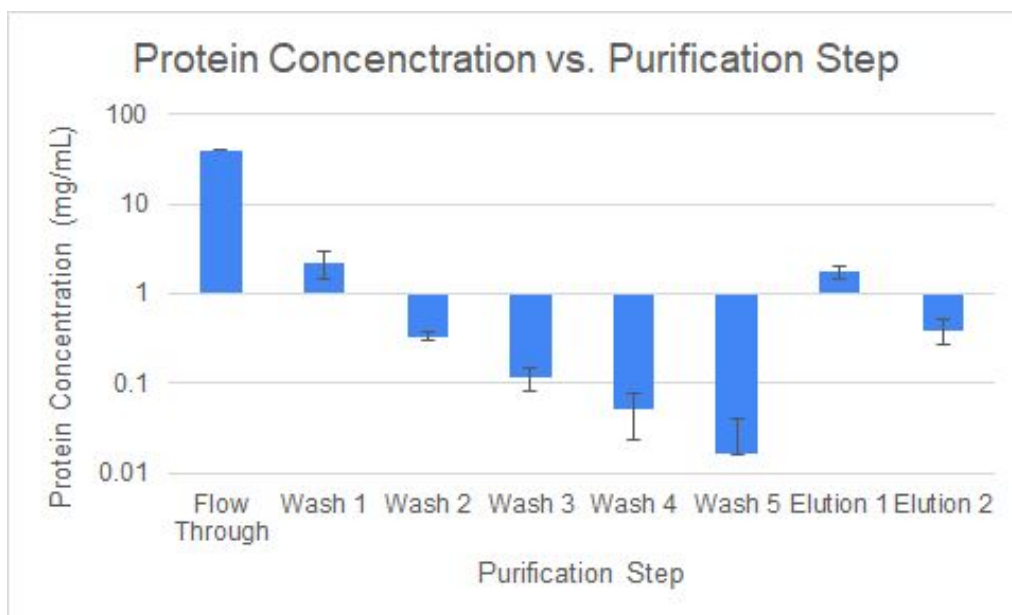


Figure 5. Average concentration of protein for each purification step. The concentration of protein present in each sample was measured using a Nanodrop Spectrometer. Only samples purified after protocol optimization were included in the average.

SrtA was successfully purified from the cell lysate collected from the *E. coli* cell pellet. The average concentration of SrtA in the first elution was 1.77 mg/mL (Fig. 5). The purity of the elution is verified by the presence of a single intense band in the elutions.

3.9 Discussion

Visual inspection of the SDS-PAGE gels shows intense bands near the 25 kDa mark in all elutions. This band is also present in most washes. The lack of bands aside from the aforementioned one in the elutions suggests that the captured protein is the modified SrtA. Also, the decreasing protein concentration in the wash steps during purification indicates non-specific products are being removed, so only the SrtA is collected during elution.

CHAPTER 4: EXTRACTION OF MAMMALIAN PROTEOME

4.1 Introduction

This stage of the project aimed to extract the total mammalian proteome from CHO cells. Both the membrane bound and cytosolic proteins were needed as the potential Molecule X.

4.2 Key Constraints

Because the proteome was going to be mixed with SrtA to capture Molecule X, the proteins needed to be functional and structurally stable. Non-denaturing conditions were necessary to isolate the proteome.

4.3 Design Description and Solutions to Problem

The Membrane Protein Extraction Kit from Biovision that was used was originally chosen to extract only the membrane bound proteins. However, the scope of this experiment was expanded to extract the entire proteome, so the protocol was modified to extract both the membrane bound and cytosolic proteins.

To release the proteins, the CHO cells were lysed with a homogenizer in a homogenization buffer (Fig. 6). Different homogenizers were used to find the most effective one.



Figure 6. Homogenizer used for mammalian proteome extraction. The cell solution was placed in the center of the homogenizer, and the handle ground up the cells to release the lysate. The homogenizer, 200 mm long by 25 mm in diameter, could process up to 7 ml of lysate.

4.4 Detailed Supporting Analysis

Originally, Molecule X was hypothesized to be a membrane bound protein to communicate with SrtA. However, research from Ren et al. showed between nitrogen-fixing Rhizobia bacteria and plant roots, tRFs are the messenger molecule¹⁵. Since tRNA resides in the cytosol, this study shows the messenger molecule does not necessarily have to be membrane bound. Therefore, both the membrane bound and cytosolic proteins were isolated as potential candidates for Molecule X.

The different homogenizers were examined to find the most efficient one for lysing. A drop of cell solution was examined under the microscope, using an intact cell nucleus as indication of unsuccessful lysing. The homogenizer that most efficiently destroyed the cell nuclei was used for all subsequent experiments.

4.5 Expected Results

After running this experiment, the expected outcome is a cell lysate containing the entire mammalian cell proteome. Specifically, both the membrane bound and cytosolic proteins should be present, and in the lysate should be Molecule X. However, because it is a signal molecule, its concentration should be relatively low.

4.6 Backup Plan

If the CHO cells were not successfully lysed through homogenization, the rapid thawing of the frozen cells would be used to lyse them. Because the cells are stored at -80 °C, exposing them to a warm water bath would crack the cell membrane, releasing the cytosolic proteins. Treating the membrane with a detergent would then release the membrane bound proteins, leading to the desired cell lysate.

4.7 Materials and Methods

4.7.1 Extraction of CHO Cell Proteome

The protocol used is adapted from BioVision, using the Membrane Protein Extraction Kit (Catalog #: K268).

The CHO cells were thawed from storage at -80 °C. They were centrifuged at 4 °C and 700 xg for 5 minutes and the supernatant was discarded. The cells were washed with 3 mL of cold bead buffer and resuspended in 2 mL of Homogenizer Buffer Mix from BioVision. The cells were homogenized on ice 30-50 times. The homogenate was transferred to a pointed tube and centrifuged at 4 °C and 700 xg for 10 minutes. The supernatant was collected.

4.8 Results

The CHO cells were successfully lysed, as verified by the homogenization efficiency under microscope. This was checked by looking for the ruptured cell membrane and nucleus. A minimum homogenization efficiency of about 70% was achieved before the mammalian proteome was collected through centrifugation.

4.9 Discussion

The cytosolic and membrane bound proteins were extracted from the CHO cells. In order to avoid protein degradation, the lysate was immediately used in subsequent cross-linking experiments.

CHAPTER 5: CROSS-LINKING

5.1 Introduction

This experiment aims to successfully capture Molecule X by cross-linking it to the purified SrtA. By combining the SrtA elution with the CHO proteome, the SrtA will bind to Molecule X. The crosslinkers will form a permanent covalent bond between the two, allowing the complex to be isolated through affinity chromatography. Two crosslinkers were identified, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and benzophenone maleimide (BPM).

5.2 Key Constraints

In order for SrtA to bind Molecule X, the buffers must emulate the native conditions and protein concentration of SrtA. Also, the buffers must be mass spectrometry compatible for later analysis through LC-MS-MS.

Also, the crosslinkers must balance strength and specificity to successfully cross-link Molecule X to SrtA. The optimal crosslinker has to be strong enough to bind Molecule X at low concentrations and specific enough to avoid non-specific binding.

5.3 Design Description and Solutions to Problem

Protocols found in articles served as a starting point to develop a protocol to cross-link SrtA and Molecule X. The article protocols detailed procedures for protein-protein cross-linking, so they had to be adapted for protein-mixture cross-linking. Also, the concentration of Molecule X was unknown, making calculations for crosslinker concentrations difficult. Changing the concentrations of the SrtA, the volume of the lysate, and the amount of crosslinker resulted in the optimal conditions for efficient binding and cross-linking.

EDC and BPM were chosen because they had a good range of strength and specificity. EDC works through nucleophilic attack, which is a weaker mechanism (Fig. 7a). EDC showed high levels of non-specific binding, which was also more difficult to wash out during purification. BPM utilizes UV-induced free radicals, which is stronger and faster (Fig. 7b). Because the reaction was quicker, there was less non-specific binding.

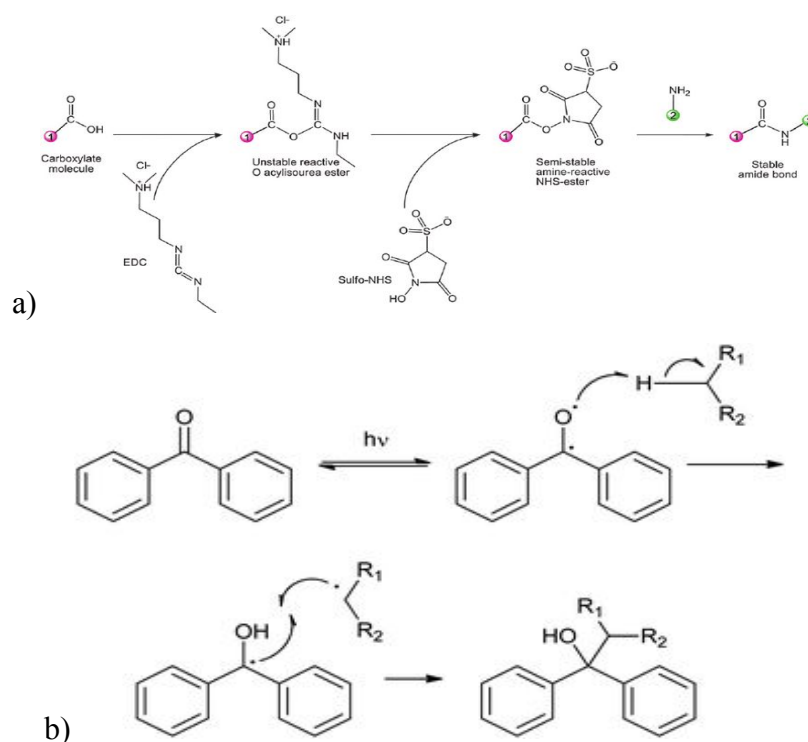


Figure 7. Crosslinker mechanisms of a) EDC and b) BPM. a) EDC reacts through nucleophilic attack. Sulfo-NHS works in conjunction with EDC to cross-link the two products together. b) BPM works through UV light induced free radicals to bond to the products.

After cross-linking, native conditions were no longer necessary. During the purification of the cross-linked complex, stronger washing and denaturing conditions could be used if necessary.

After initial cross-linking trials with EDC and BPM, the purification protocol was optimized to reduce non-specific binding and increase the purity of the Molecule X-SrtA complex. Glycerol, 2-mercaptoethanol, and an increased concentration of NaCl was added to the buffers. Glycerol helps stabilize the proteins upon binding to the Ni-NTA beads, and 2-mercaptoethanol reduces disulfide bonds. The increased NaCl concentration helps to reduce non-specific binding and remove unwanted products from the beads. Also, the number of wash steps was increased from 5 to 7, which further removes non-specific products from the beads.

5.4 Detailed Supporting Analysis

Multiple trials of each crosslinker were run, and the results from the SDS-PAGE analysis were used to change the calculations for later reactions. BPM showed promise early during the trials, so it was used for all subsequent cross-linking experiments.

5.5 Expected Results

SDS-PAGE gel was used to analyze the results of each cross-linking experiment. Because the molecular weight of Molecule X was unknown, the indicator for successful cross-linking was a distinct band in the elutions, but not the washes, that was also larger than 21.7 kDa.

5.6 Backup Plan

If both EDC and BPM were unsuccessful, other crosslinkers with varying strength and specificity would be used. Dibromobimane and 4-benzoylbenzoic acid, succinimidyl ester were identified as potential backup crosslinkers.

5.7 Materials and Methods

5.7.1 EDC Cross-linking

The protocol used was adapted from the Thermofisher NHS and Sulfo-NHS Manual¹⁷.

Activation buffer (100 mM MES, 500 mM NaCl, pH 6) was added to SrtA to achieve a 1 mL solution with SrtA concentration of 1 mg/mL. EDC from Sigma-Aldrich was added to reach a final concentration of 2 mM, which results in a 10-fold molar excess of EDC to SrtA. N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS) from Sigma-Aldrich was added to the reaction mixture to reach a final concentration of 5 mM. The reactants were mixed and allowed to react at room temperature for 15 minutes. The EDC was inactivated with 2-mercaptoethanol at a final concentration of 20 mM. Concentrated bead buffer was added to raise the pH above 7.0, then the homogenate was added to the reaction solution. The solution was mixed and reacted at room temperature for 2 hours. The reaction was quenched by adding hydroxylamine at a final concentration of 10 mM.

5.7.2 BPM Cross-linking

The protocol used was adapted from the article Crosslinking of low-affinity glycoprotein ligands to galectin LEC-1 using a photoactivatable sulfhydryl reagent¹⁸.

2.5 µg SrtA was suspended in reaction buffer (bead buffer with 1 mM EDTA, pH 7.2) at a concentration of 1 mg/mL. 100 µM 4-(N-maleimido) benzophenone (BPM) from Sigma-Aldrich was dissolved in dimethylformamide. 0.2 µL BPM solution was added to the SrtA solution and incubated in the dark at room temperature for 30 minutes. The unreacted BPM was quenched with 0.2 µL of 1 M DTT. The homogenate was added to the SrtA solution and irradiated with 365 nm light at room temperature for 3 minutes.

5.7.3 Purification of Cross-linked Products

The supernatant from the cross-linked solution was added to the clean Ni-NTA beads and rocked at 4 °C for 2 hours. The binding mixture was centrifuged at 4 °C and 2500 rpm for 1 hour. The flow through supernatant was collected and 5 mL of wash buffer (50 mM Tris-HCl, 700 mM NaCl, 0.5 mM CaCl₂, 5 mM 2-mercaptoethanol, 20 mM imidazole, and 0.09 mM PMSF, pH 8) was added to the bead solution. The solution was rocked at 4 °C for 20 minutes then centrifuged at 4 °C and 2000 rpm for 5 minutes. The wash supernatant was collected, and the bead solution was washed 4 more times. The beads were eluted with elution buffer (50 mM Tris-HCl, 200 mM NaCl, 0.5 mM CaCl₂, 5 mM 2-mercaptoethanol, 250 mM imidazole, and 0.09 mM PMSF, pH 8). First 2 mL of the elution buffer was added and the solution rocked at 4 °C for 20 minutes. The solution was centrifuged at 4 °C and 2000 rpm for 5 minutes and the first elution supernatant was collected. Then 1.5 mL of the elution buffer was added and the solution rocked at 4 °C for 20 minutes. The solution was centrifuged at 4 °C and 2000 rpm for 5 minutes and the second elution supernatant was collected. The flow through, wash supernatants, and elution supernatants were stored at -20 °C.

5.7.4 Running SDS-PAGE Gel

For the flow through, the 5 washes, and the 2 elutions, 48 μ L of each sample was mixed with 12 μ L of 5X loading dye in an Eppendorf tube. A gel from GenScript was attached to the Invitrogen SDS-PAGE apparatus and the container was filled with GenScript running buffer. 10 μ L of Thermofisher 26628 protein ladder and each sample was loaded into the gel and ran at 100 V for 1.5 hours.

5.7.5 Silver Staining

Refer to protocol provided in PierceTM Silver Stain for Mass Spectrometry from ThermoScientific (Ref 24600).

5.8 Results

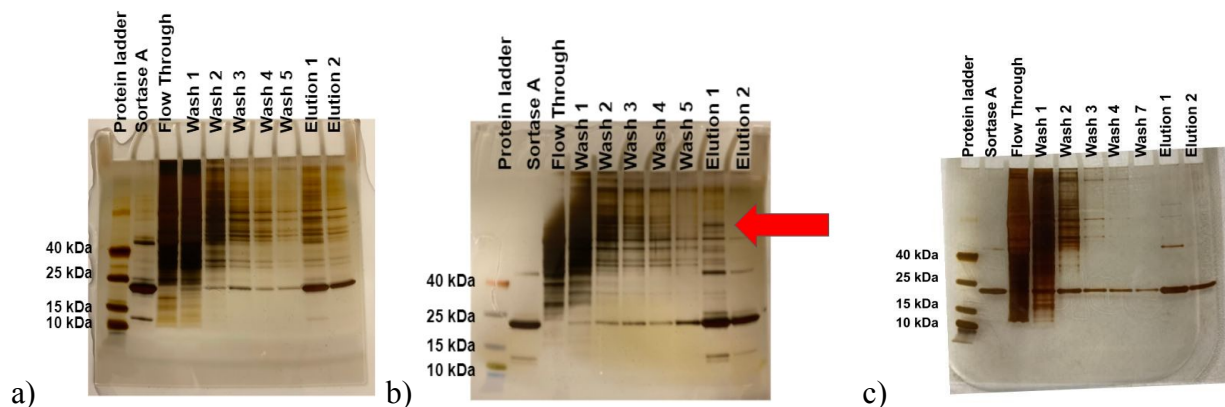


Figure 8. SDS-PAGE gels of a) initial EDC cross-linking, b) initial BPM cross-linking, and c) optimized BPM cross-linking. The elution columns contain the purified samples after cross-linking and should contain Molecule X. The red arrow in b) emphasizes the band in the elution that is not present in the washes, which makes it a strong candidate for Molecule X.

SilverStained gels from both cross-linking methods showed numerous bands in the elutions. The elutions from the BPM had a higher number of defined bands while having less bands in total than EDC. Because silver staining is a more sensitive stain, more products, even those in small concentrations, appear on the gel. Because Molecule X is hypothesized to be present in low quantities, the silver stain is used. The SrtA used in the cross-linking for the initial

trials (Fig. 8 a&b) was purified using an older protocol that did not include glycerol in the wash buffers. This contributed to the higher amount of impurities seen.

5.9 Discussion

Both also showed bands around the 25 kDa marker suggesting that the conditions and procedures for binding to the Ni-NTA beads were working as desired. Most of the bands in the elutions were also present in the washes, indicating they were non-specific products. However, the first silver stained gel from the BPM trial (Fig. 8b) showed a band around 50 kDa in the elution that was not in the washes. This band suggested the Molecule X-SrtA complex was successfully cross-linked and purified. No such band was visible in the EDC trial. All subsequent cross-linking trials used BPM because it was best performing crosslinker. The determination of band intensity and subsequent categorization of which bands contained the cross-linked products was based on semi-quantitative observations. Furthermore, since the native concentration of Molecule X is unknown, even the faintest of bands should not be ruled out. These limitations contributed to the decision to perform the mass spectrometry analysis using the liquid elution samples instead of only testing specific bands with in-gel digestion.

CHAPTER 6: DIGESTION AND MASS SPECTROMETRY ANALYSIS

6.1 Introduction

This experiment identifies the molecule cross-linked to SrtA and was performed by MtoZ Biolabs. Once the samples of the Molecule X-SrtA complex were purified, they were digested using trypsin. The resulting peptide fragments were analyzed using LC-MS-MS and compared to a proteomics database using Maxquant.

6.2 Key Constraints

The budget allowed for 3 samples and a control to be analyzed. Based on the early successes from the BPM cross-linking experiments, only samples cross-linked with BPM were analyzed. Even though the samples were not pure, by analyzing 3 samples, the data from each sample can be compared and cross-referenced to find Molecule X.

6.3 Design Description and Solutions to Problem

Trypsin cleaves peptide bonds at lysine and arginine residues. Because the digestion is predictable, each protein has a unique fingerprint of resulting peptides. These peptide fragments are compared to the proteomics database to align the peptide fragments in the sample solution with known protein sequences.

6.4 Detailed Supporting Analysis

MtoZ Biolabs calibrates their mass spectrometer to effectively analyze the proteins in the sample solution. The samples were prepared to cleave any disulfide bonds and denature the proteins.

6.5 Expected Results

As part of the LC-MS-MS analysis, MtoZ Biolabs provides a report of the proteins present in each solution. Proteins found in the control are excluded from the other samples, and

the remaining proteins of each sample are compared to each other. The probable candidate for Molecule X should be present in multiple samples.

6.6 Backup Plan

No one sample was guaranteed to contain Molecule X, so 3 samples were analyzed and cross-referenced with each other.

6.7 Materials and Methods

The protocol is provided by MtoZ Biolabs.

6.7.1 Chemicals and Instrumentation

DL-dithiothreitol (DTT), iodoacetamide (IAA), formic acid (FA), acetonitrile (ACN), methanol, were purchased from Sigma (St. Louis, MO, USA), trypsin from bovine pancreas was purchased from Promega (Madison, WI, USA). Ultrapure water was prepared from a Millipore purification system (Billerica, MA, USA). An Easy-nLC1000 system coupled with a LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, USA) with an ESI nanospray source.

6.7.2 Sample Preparation

Transfer the solution into Microcon devices YM-10 (Millipore). The device was centrifuged at 12,000g at 4°C for 10 min. Subsequently, 200 µL of 50 mM ammonium bicarbonate were added to the concentrate followed by centrifugation and repeat once. After reduced by 10 mM DTT at 56°C for 1 h and alkylated by 20 mM IAA at room temperature in dark for 1h, the device was centrifuged at 12,000g at 4°C for 10 min and wash once with 50 mM ammonium bicarbonate. Add 100 µL of 50 mM ammonium bicarbonate and free trypsin into the protein solution at a ratio of 1:50, and the solution was incubated at 37°C overnight. Finally, the device was centrifuged at 12,000g at 4°C for 10 min. 100 µL of 50 mM ammonium bicarbonate was added into the device and centrifuged, and then repeated once. Lyophilize the extracted

peptides to near dryness. Resuspend peptides in 2-20 μ L of 0.1% formic acid before LC-MS/MS analysis.

6.7.3 nanoLC

Nanoflow UPLC: EUltimate 3000 (ThermoFisher Scientific, USA); Nanocolumn : 100 μ m \times 15 cm in-house made column packed with a reversed-phase ReproSil-Pur C18-AQ resin (3 μ m, 120 Å, Dr. Maisch GmbH, Germany); Loaded sample volume: 5 μ L. Mobile phase: A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile. Total flow rate : 300 nL/min. LC linear gradient: from 2% to 2% B for 10 min, from 2% to 35% B for 90 min, from 35% to 80% B for 1 min, from 80% to 80% B for 14 min and from 80% to 2% B for 1 min.

6.7.4 Mass spectrometry

Q ExactiveTM HF-X Hybrid Quadrupole-OrbitrapTM Mass Spectrometer (Thermo Fisher Scientific, USA). Spray voltage: 2.2 kV. Capillary temperature: 270 °C. MS parameters: MS resolution: 60000 at 400 m/z. MS precursor m/z range: 400.0-1600.0.

MS/MS parameters: Product ion scan range: start from m/z 100. Activation Type: HCD. Min. Signal Required: 1500.0. Isolation Width: 3.00. Normalized Coll. Energy: 40.0. Default Charge State: 6. Activation Q: 0.250. Activation Time: 30.000. Data dependent MS/MS: up to as much most intense peptide ions from the preview scan in the Orbitrap.

6.7.5 Data analysis

The 4 raw MS files were analyzed and searched against a protein database based on the species of the samples using Maxquant (1.6.2.10). The parameters were set as follows: the protein modifications were carbamidomethylation (C) (fixed), oxidation (M) (variable); the enzyme specificity was set to trypsin; the maximum missed cleavages were set to 2; the precursor ion mass tolerance was set to 20 ppm, and MS/MS tolerance was 20 ppm. Only high confident identified peptides were chosen for downstream protein identification analysis.

6.8 Results

Include processed data chart w protein ID, name, size, known subcell location, and molecular and cellular functions along with peak intensity stuff

The samples were sent to MtoZ BioLabs for mass spectrometry analysis where they were successfully digested using trypsin. The resulting data was sent back. Refer to Appendix C for the full tables.

6.9 Discussion

Because samples that were analyzed were the elutions for each cross-linking trial, the mass spectrometer identified all proteins present in the solution, including non-specific products. Therefore, the data was processed to eliminate products present in the control. The remaining candidates were analyzed to determine the identity of Molecule X.

CHAPTER 7: SUMMARY AND CONCLUSION

7.1 Project Summary

The 5 major experiments of this project resulted in proteomics data detailing the proteins present after cross-linking and purification (see Appendix C). The data was processed to discern candidates for Molecule X. Candidate groups were formed to cluster similar proteins together, using the similarities to inform how Molecule X functions and a likely identity.

One candidate group is the duplicates between samples. Proteins present in the control (Sample 4) were eliminated from Samples 1-3, and proteins present in multiple samples were grouped together. One hypothesis is Molecule X should be captured in each trial, so it should present in at least more than one sample. This group includes Pentatricopeptide repeat-containing protein 1 (PTCD1), obscurin, and mortality factor 4-like protein 2 (MORF4L2).

Other candidate groups include proteins dependent on calcium ions, membrane associated proteins, GPCR associated proteins, and ATP or GTP binding proteins. The proteins in each candidate group were searched for using current literature to understand how they function and the possibility of acting as Molecule X. Likely candidates will be verified in later experiments.

7.2 Evaluation of Milestones

The protocols developed during this project lead to the successful capture of Molecule X. First, SrtA was successfully produced in *E. coli* cells as evidenced by the bacterial growth in the presence of kanamycin in both the plating agar and LB broth.

SrtA was then purified from the bacterial cells. The purification protocol was optimized to improve purification efficiency, as shown by the single, intense bands in the SDS-PAGE gels.

The entire mammalian proteome was extracted from CHO cells. Both the membrane and cytosolic proteins were collected to give the best chance of finding Molecule X. Successful homogenization was verified through visual inspection of homogenization efficiency.

The solutions of SrtA and CHO proteome were mixed. The added crosslinkers created a covalent attachment between SrtA and Molecule X, allowing the purification of the complex. SDS-PAGE analysis revealed the presence of the complex in the collected solution.

The purified complex was digested and analyzed using mass spectrometry. These results were analyzed to identify candidates for Molecule X. These candidates will be verified in future experiments.

7.3 Future Work

Image J may be used to quantify the band intensities and homogenization efficiencies to better verify the completion of each experiment. A more quantitative approach would be useful for targeted proteomics, where Molecule X is being purified rather than identified. Future experiments should also include the quantification of the native concentrations of Molecule X and the kinetics of the SrtA-Molecule X interaction.

Once candidates for Molecule X are identified, an siRNA experiment will verify if the protein acts as Molecule X. siRNA will knock out the gene encoding for the Molecule X candidate, so the cells will not produce that protein. Since Molecule X activates bacterial cells to infect the mammalian cells, the cells with Molecule X knocked out will not be infected by bacteria. This experiment will show if the candidate protein is Molecule X.

After the siRNA experiment, an immunoassay will further verify the function of Molecule X. Similar to the siRNA experiment, cells treated with an antibody antagonistic to Molecule X will not be infected by bacteria. If successful, the antibody can be used towards development of an anti-infective immunotherapeutic.

CHAPTER 8: ENGINEERING STANDARDS AND REALISTIC CONSTRAINTS

8.1 Economic

8.1.1 Cost of Development

This project had a total budget of \$1,200 to cover all materials and travel costs (refer to section 1.8). A 2010 study estimated the average cost of developing a monoclonal antibody (mAb) drug to be roughly \$1.8 billion. This figure covers from the discovery through drug development phases (Fig. 9)^{19,20}.

Discovery and Development Phase	Out-of Pocket Costs (\$-millions)	Total Cost (Capitalized at 11%, \$-millions)
Target-to-hit	\$24	\$94
Hit-to-lead	\$49	\$166
Lead optimization	\$146	\$414
Preclinical	\$62	\$150
Phase I	\$128	\$273
Phase II	\$185	\$319
Phase III	\$235	\$314
Submission to launch	\$44	\$48
TOTAL:	\$873	\$1,778

Figure 9. Breakdown of discovery and development costs from the 2010 Eli Lilly study. The costs are discounted taking the year of FDA approval as the base year for discounting. Eleven percent is a typical interest rate used by the pharmaceutical industry to discount costs. The costs here are for both small molecule and biological new molecular entities (NMEs), defined as chemically unique pharmaceuticals that have not yet been marketed in the U.S. in any form.

The cost of developing a vaccine from the preclinical phase to the clinical 2a phase can be anywhere in the range of \$14-159 million if assuming no risk of failure and \$137 mill-1.1 billion when accounting for failures²¹.

8.1.2 Cost of Ownership

Drug targets cannot be owned and this project was not meant to produce any physical or ownable delivery. Molecule X can be used to conduct research that would result in drugs or delivery systems that can be owned. Production of such drugs can have varying costs depending on who is developing them and at what scale. A report from Biopharm states that current stainless steel technology would require a minimum of \$300 million capital investment to set up bioreactors that would be able to handle the production scale necessary for the market. This amount does not include maintenance or operating costs²².

8.1.3 User's Perspective

The current patient cost of a relatively high-dose antibiotic regimen is \$50-120 per day²³. The cost of treating an antibiotic resistant infection on average is just under \$30,000 per case¹⁶. The average cost of mAb treatment is ~\$100,000 annually, though this figure is skewed by the higher costs of oncology and hematology related mAbs²⁴.

8.1.4 Is it Worth it?

Novel treatments are only feasible if they are just as if not more affordable than the existing treatments. The cost of developing a drug is far outweighed by the national cost of current treatments, but the average patient costs would likely increase if the novel drug is an mAb.

8.2 Sustainability

8.2.1 Why Sustainability?

Drug discovery, development, and production are all costly and intensive processes. While the scope of this project does not cover these stages, they are the natural next steps. In addition to being monetarily expensive, they are also costly in terms of materials and energy. Since drug based research is a vital component of the constantly evolving field of medicine, these steps have and will continue to be done numerous times. The sheer amount of resources used mandates that sustainability be considered. Three fundamental approaches that can be taken

to help improve the sustainability of an engineered product are to limit growth, to use non-renewable resources more carefully, and to develop renewable resources. The limitation of drug development would be ill-advised, so focusing on reducing the amount of non-renewable resources that are used and finding as many alternatives for said resources is vital.

8.2.2 Material Resources

The materials used in this project are largely non-renewable as the cells, buffers, and reagents are considered consumable. In order to preserve the integrity and reliability of the data, the methods of obtaining these materials should not be tampered with.

8.2.3 Energy Resources

The main sources of energy consumption for this project are the operation of devices critical to a standard lab and the transportation of ordered materials. In a small-scale research lab, the general energy consumption is low in comparison with larger facilities which makes it hard to optimize. This could be considered more fully on a production scale at a large pharmaceutical company. Some current methods to help improve production efficiency include running tests in parallel and making larger batches.

8.2.4 Sustainable Engineering

Multiple protocol alterations were made during this project for the purpose of reducing waste and increasing efficiency. In order to operate on a need basis only, the amount of buffer and waste created was reduced by using the same original buffer for all purification steps. This was done by aliquoting out the necessary amount and making the necessary additions as each step mandated. This reduced the amount that was used by over 50%. Similarly, multiple trials were often run in parallel so that the energy consumed in running a single test instead was enough to run multiple trials. Increasing the efficiency of the procedures both saved in time and effort as well as materials and energy.

8.3 Ethics

8.3.1 Ethical Approaches

Though seemingly straightforward, there are many factors that must be considered in deciding if this research and the subsequent work is ethical. Due to the more universal nature of medicine, a utilitarian approach fits best. This approach entails weighing the potential good that this project can do against the costs that must be met to accomplish said good. For this project, the good would be the lives saved and suffering averted by developing drugs to treat drug-resistant microbes while the costs would include the time, labor, and resources used to make said drug a reality. Furthermore, this approach requires trying to conceptualize a number of unknown factors such as the monetary gains and losses and the societal impacts.

8.3.2 Framework for Thinking Ethically

Regardless of the ethical approach used, the steps undertaken to analyze the issue are largely the same. These steps include identifying the issue, gathering information about the issue, determining the possible routes that can be taken, making a decision, and executing the decision.

8.3.3 Ethical Considerations

For this project, the issue is determining whether or not this line of reasoning should be explored. Is it worth the time, money and resources when considering the probability of successfully developing a usable drug and the scope in which this drug can do good? Are the current treatments or other fields of research sufficient enough to make this project obsolete? Will any resulting products be accessible enough to actually benefit society as a whole? These questions must then be weighed with the knowledge of which parties have a genuine stake in the issue. Manufacturers have a large stake in the economic aspects and will likely only continue the research if it can be made profitable. Patients are the people most directly affected, and due to the nature of infections, everyone is a potential patient. It is important to note that people spending more time in hospitals are more likely to develop antibiotic resistant infections. Furthermore, everyone has a stake in the effect of antibiotics on the environment. This includes the disruptions to ecosystems and increased likelihood of developing superbugs in areas that are

constantly exposed to antibiotic waste²⁵. Some additional considerations are the sourcing of materials and methods of testing future drugs.

Now that the issue and factors surrounding it have been considered, the potential responses must be formulated. The two main choices are to continue the research and move into validation and drug discovery or to stop any further experiments. Since the current research is not yet advanced enough to warrant any immediate concerns about the theoretical impacts of continuing this research, utility ethics would mandate moving forward. All possible benefits and concerns are purely speculation at this point. Drug target identification is the first step in the long process of drug development and there is no need as of yet to halt the process. As long as safety and efficacy guidelines are followed and data is presented in an objective, unbiased way, future research will be able to give more accurate information about this issue.

8.4 Health and Safety

8.4.1 Engineering Failures

All projects have the potential to fail. Engineering projects in particular have the potential to be devastating when they fail. In order to safeguard against this, the sources of failure must be identified and accounted for. Some of the most common reasons for failure are the materials, the design, environmental factors, human error, or a combination of these. In this project, some of the most likely sources of failure are design flaws and human error. This could be due to the hypothesis being incorrect or from errors in the procedures.

8.4.2 Engineering for a Healthier and Safer World

The overarching goal of engineering is to make the world safer and healthier. In order to accomplish this, measures such as better engineering, government regulation, and poke yoke (mistake proofing) must be implemented into how a project is designed and executed. Going forward, potential measures in each category to be integrated to help prevent failure. Better engineering would entail doing even more trials and background research to solidify the assumptions that the hypothesis makes and optimizing the experimental protocols. Government regulation would come in the form of the FDA regulations that need to be met before a drug can

be put on the market²⁶. Poke yoke would similarly be enhanced by the development of standard procedures that are used when producing drugs to ensure quality control.

8.4.3 Unanticipated Consequences

The potential impacts of this project implies a large range of potential unanticipated consequences. At this point, the actual mechanism of action that any resulting drug would have is unknown. This means that the potential side effects of a drug are also unknown at this time. Similarly, the hypothesis that SrtA is the only receptor used by bacteria and that blocking it will prevent infection could be incorrect, thus making an effective drug from the data gathered would be unlikely. Another potential consequence that is hard to predict are the economic impacts. A drug developed from Molecule X could end up replacing the current cost of fighting antibacterial infections with an even higher cost. It is already known that antibody drugs can be extremely costly to produce and to purchase as a consumer, so this drug could end up only being given to the rich, further increasing the disparity in healthcare quality between socioeconomic classes²⁴. A consequence that would only be seen over the course of multiple years would be the environmental impact of a drug. If antibiotic waste continues at the same rate, then adding another drug could simply provide even more pressure for bacteria to mutate and become superbugs.

8.4.4 In Defense of Failure

Regardless of the potential consequences of a hypothetical drug that might be developed down the line, this project, even if it is later found that the hypothesis is false, is still important. Anti-infectives are largely unexplored and any research made into the field is valuable. If Molecule x does interfere with a pathway that would make it unsafe to block, then it both eliminates a potential drug target and provides more information about said pathway. This knowledge would also guide future research to look more at SrtA itself as a potential target. Similarly, any economic impact that this could have can be used to improve the current public health system. Any failure, whether it be a technical failure or a failure of implementation, can be used to improve and make more informed decisions in the future.

8.4.5 Risks

This project has limited risks aside from those common to working in a wet lab environment. These are addressed by following the recommended guidelines for personal protective equipment (PPE) and by working in pairs. The main source of risks comes from any potential drugs developed using the results of this project. The acceptable level of risk and guidelines for how to quantify said risk is determined by FDA standards. These government approved regulations require extensive testing of any potential drug before it is used on humans or released to the public.

8.5 Social

8.5.1 Role of Technology in Society

Technology has and continues to transform society. In an unending cycle, technology evolves in response to societal needs, and medicine is no different. As a vital part of modern society, medicine is both a large reason for the steadily rising length and quality of life, but it has also enabled crises such as the opioid epidemic and the rise of infectious superbugs. While medicine addresses many life threatening diseases and conditions, no treatment is risk free, whether the risk comes from side effects or the potential for abuse. In the case of the medicine that this project could birth, the antibiotic resistance crisis could be averted, but it could also exacerbate the crisis by creating a new form of resistance and feeding into the current economic inequality.

8.5.2 Deciding What is to be Done

In deciding whether or not to pursue this line of research in drug development, the aforementioned benefits and consequences need to be weighed. Will these drugs improve society and the overall quality of human life, or will they create more problems than they solve? While this is impossible to truly know, something that is inevitable is progress. Need is the mother of invention, so the need for better drugs to treat infections necessitates a solution. New drugs are

constantly being created, tested, and used. Ultimately, any further research done on this subject, whether it disproves this project or not, furthers understanding and knowledge of the field.

8.5.3 The Role of the Engineer

In carrying out research, it is the engineer's responsibility to ensure that the proper measures are taken to prevent as many negative consequences as possible. In this case, that means doing one's due diligence in ensuring that the background research and project design are sound and abiding by the appropriate guidelines to ensure safety and efficacy. All data and the presentation of the results should be truthful and unbiased. This even extends to being willing to stop the project if it proves to be harmful or the hypothesis is proven false.

8.5.4 Human Needs

In order to improve the quality of life, a product must meet at least one of the major human needs. In the case of medical advances, the aforementioned need fits within the realm of safety. Medicine can help the body and mind, making it vital in achieving one of the most basic human needs.

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APPENDICES

Appendix A: Tables

Table 1. *E. coli* cell pell masses after culturing and protein induction. Cell pellets were collected through centrifugation.

Date	<i>E. coli</i> Cell Pellet Mass (g)
1/21/2020	7.3
1/28/2020	9.3
3/10/2020	11.4
Average	9.3

Table 2. Protein concentrations for each SrtA purification step. Concentrations were measured using nanodrop spectrometry.

	Protein Concentration (mg/mL)							
Date	Flow Through	Wash 1	Wash 2	Wash 3	Wash 4	Wash 5	Elution 1	Elution 2
1/14	39.7	2.73	0.310	0.093	0.032	0.00	1.985	0.487
1/30	-	1.67	0.358	0.143	0.070	0.033	1.548	0.304
Average	39.7	2.20	0.334	0.118	0.051	0.017	1.77	0.396

Table 3. Proposed Project Budget

Item	Cost	Funding From
Consumables	\$125	
Gloves	\$10	Asking Undergraduate Programs to fund
Wipes	\$10	Asking Undergraduate Programs to fund
Pipetmen Tips	\$60	Asking Undergraduate Programs to fund
Cuvettes	\$30	Asking Undergraduate Programs to fund
Cell Culture Plates	\$15	Asking Undergraduate Programs to fund
Chemicals	\$600	
Reagents	\$250	Asking Undergraduate Programs to fund
Crosslinkers	\$175	Asking Undergraduate Programs to fund
Buffer Components	\$75	Asking Undergraduate Programs to fund
Ni-NTA Beads	\$100	Asking Undergraduate Programs to fund
Biological Components	\$8,175	
Bacterial Cells	\$450	Available from Dr. Zhang
Mammalian Cells	\$7,000	Available through JOINN
Extraction Kit	\$410	Asking Undergraduate Programs to fund
Trypsin	\$65	Asking Undergraduate Programs to fund
Plasmids	\$250	Available from Dr. Zhang
Misc.	\$1,240	
LC-MS-MS	\$1,000	Available through JOINN
Travel	\$240	Asking Xilinx Grant to fund
Total	\$10,140	
Amount Requested	\$1,200	

Table 4. Project Budget

Item	Cost	Funding From
Consumables	\$0	Available through the Zhang Lab
Chemicals	\$240	
Biological Components	\$0	Available through the Zhang Lab
LC-MS-MS	\$1,000	
Total	\$1,240	

Appendix B: Additional Crosslinker Gels

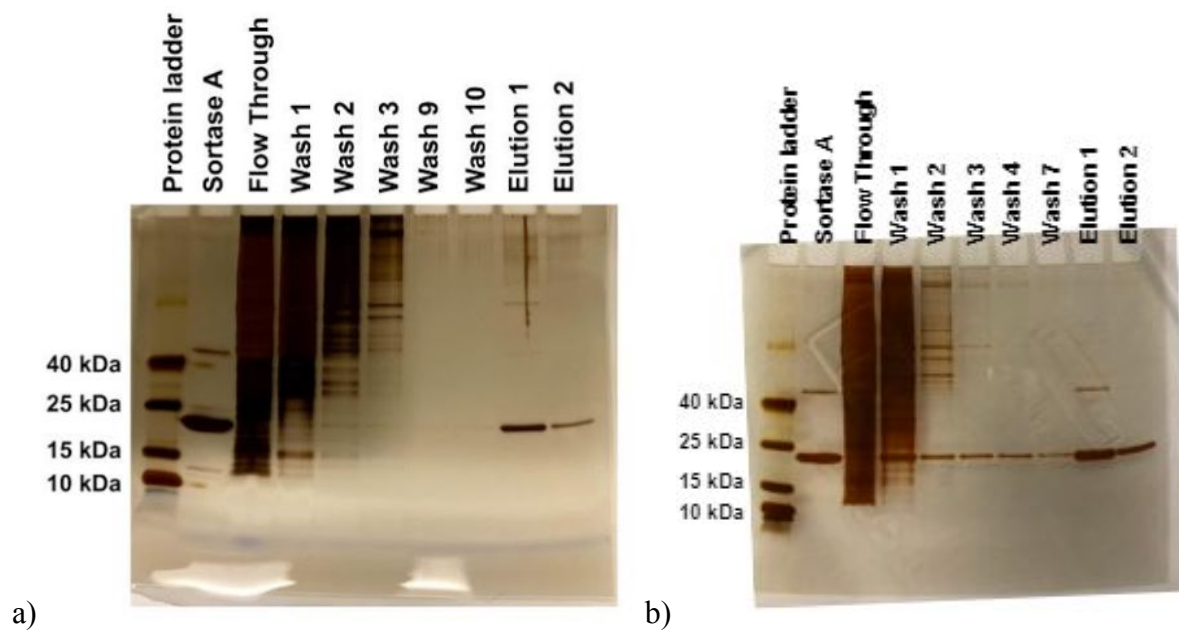


Figure 10. SDS PAGE gels of BPM cross-linking trial on a) 2/25 and b) 3/6.

Appendix C: LC-MS-MS Data

a) Sample 1 (BPM Trial 1/16)

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	Protein ID	Majority p	Protein na	Gene nam	Fasta heac	Number o	Peptides	Unique pe	Sequence	Unique se	Mol. weig	Sequence le	Sequence	Q-value	Score	Intensity
2	P0A9K9	P0A9K9	FKBP-type slyD		sp P0A9K9	1	4	4	54.6	54.6	20.853	196	196	0	323.31	941380000
3	P0CE48;P	P0CE48;P	Elongator tufB;tufA		sp P0CE48	3	12	12	36.8	36.8	43.313	394	394;394;9	0	323.31	1452200000
4	P0A6Y8	P0A6Y8	Chaperon dnaK		sp P0A6Y8	1	10	10	26.3	26.3	69.114	638	638	0	145.6	401110000
5	P0A6Z6	P0A6Z6	Nickel-res nikR		sp P0A6Z6	1	5	5	54.9	54.9	15.094	133	133	0	78.854	427760000
6	P63284	P63284	Chaperon clpB		sp P63284	1	4	4	8.4	8.4	95.584	857	857	0	58.981	67338000
7	P0A9M8	P0A9M8	Phosphate pta		sp P0A9M8	1	4	4	8	8	77.171	714	714	0	43.77	42256000
8	P64588	P64588	Transcript yjiI		sp P64588	1	4	4	17.4	17.4	23.401	207	207	0	40.311	206790000
9	P0A6F3	P0A6F3	Glycerol ki gfpK		sp P0A6F3	1	3	3	8.8	8.8	56.23	502	502	0	28.885	26302000
10	G3I806;REV	G3I806;REV	G3GRW5		tr G3I806	2	2	2	11.8	11.8	8.1435	68	68;1195	0	26.805	43278000000
11	G3H5K9	G3H5K9			tr G3H5K9	1	1	1	1.5	1.5	65.703	602	602	0	25.005	139350000
12	G3HIP9	G3HIP9			tr G3HIP9	1	1	1	0.5	0.5	128.02	1148	1148	0	23.625	100510000
13	P0C8J8	P0C8J8	D-tagatose gatZ		sp P0C8J8	1	2	2	14.8	14.8	47.108	420	420	0	20.011	24799000
14	P0A8B4	P0A8B4	ATP synth atpD		sp P0A8B4	1	2	2	6.7	6.7	50.325	460	460	0	19.55	6186400
15	P0A853	P0A853	Tryptophan tnaA		sp P0A853	1	3	3	10	10	52.773	471	471	0	19.438	62613000
16	P0A705	P0A705	Translation infB		sp P0A705	1	2	2	2.1	2.1	97.349	890	890	0	19.057	34877000
17	P77398	P77398	Bifunction amnA		sp P77398	1	3	3	7.1	7.1	74.288	660	660	0	18.282	26795000
18	P0A7E5	P0A7E5	CTP synth pyrG		sp P0A7E5	1	3	3	5.7	5.7	60.373	545	545	0	18.059	5590500
19	G3IJZ5	G3IJZ5			tr G3IJZ5	1	1	1	6.5	6.5	13.575	124	124	0	17.584	31591000
20	G3HX10	G3HX10			tr G3HX10	1	1	1	2.3	2.3	33.53	303	303	0	17.022	55130000
21	G3GU88	G3GU88			tr G3GU88	1	1	1	1.3	1.3	65.307	547	547	0	16.548	4468500000
22	G3HAC6	G3HAC6			tr G3HAC6	1	1	1	0	0	4007	36026	36026	0	15.879	25300000
23	P0A6P1	P0A6P1	Elongator tsf		sp P0A6P1	1	2	2	9.2	9.2	30.423	283	283	0	14.504	19390000
24	G3HHM5	G3HHM5			tr G3HHM5	1	1	1	0.1	0.1	760.28	6993	6993	0	13.536	27118000000
25	P37440	P37440	Oxidoreduc ucpA		sp P37440	1	1	1	4.6	4.6	27.85	263	263	0	13.532	0
26	P0AG30	P0AG30	Transcript rho		sp P0AG30	1	2	2	5	5	47.004	419	419	0	12.511	18898000
27	G3HLL7;G	G3HLL7;G	G3IIF5;G3IIO7;G3IH98		tr G3HLL7	4	1	1	2.6	2.6	49.075	427	427;431;4	0	12.291	0
28	P69924	P69924	Ribonuclei rrdB		sp P69924	1	1	1	2.1	2.1	43.517	376	376	0	9.3738	28213000
29	G3GTC6	G3GTC6			tr G3GTC6	1	1	1	1.9	1.9	47.094	421	421	0	9.2509	10040000000
30	P0A7G6	P0A7G6	Protein Rec recA		sp P0A7G6	1	1	1	3.1	3.1	37.973	353	353	0	9.2205	11899000
31	G3H180	G3H180			tr G3H180	1	1	1	0.4	0.4	254.48	2318	2318	0	8.8604	2719800000
32	P0A6H5	P0A6H5	ATP-deper hslU		sp P0A6H5	1	1	1	2.3	2.3	49.593	443	443	0	8.4913	7276600
33	P0A9A6	P0A9A6	Cell divisio ftsZ		sp P0A9A6	1	1	1	5	5	40.323	383	383	0	8.046	6205200
34	G3GX13	G3GX13			tr G3GX13	1	1	1	4	4	19.006	174	174	0	7.5771	999850000
35	G3I7A0;G	G3I7A0;G	G3HHM5;Q3HR12		tr G3I7A0	3	1	1	5.7	5.7	18.172	157	157;302;3	0	7.3648	242060000
36	G3IGX6	G3IGX6			tr G3IGX6	1	1	1	0.7	0.7	156.37	1381	1381	0	7.2136	0
37	G3H666	G3H666			tr G3H666	1	1	1	2	2	89.064	841	841	0	7.064	59117000
38	G3IBN8;G	G3IBN8;G	G3IKD4;G3IKI7;G3IBN9		tr G3IBN8	4	1	1	2.6	2.6	48.528	457	457;531;6	0	6.9023	16496000
39	P0A9S3	P0A9S3	Galactitol- gatD		sp P0A9S3	1	1	1	3.2	3.2	37.39	346	346	0	6.8037	10460000
40	G3HEX9	G3HEX9			tr G3HEX9	1	1	1	2.3	2.3	56.673	488	488	0	6.5655	1058500000
41	P36683	P36683	Aconitate acnB		sp P36683	1	1	1	1.3	1.3	93.497	865	865	0	6.5425	0
42	G3HF72	G3HF72			tr G3HF72	1	1	1	1.4	1.4	77.902	691	691	0	6.5042	0
43	G3I2M7	G3I2M7			tr G3I2M7	1	1	1	0.8	0.8	104.54	945	945	0	6.3624	9320500
44	G3HJ46	G3HJ46			tr G3HJ46	1	1	1	17.7	17.7	11.446	96	96	0	6.3191	2464500
45	P0A6F5	P0A6F5	60 kDa chi- groL		sp P0A6F5	1	1	1	3.1	3.1	57.328	548	548	0	6.2841	6544800
46	G3HK54	G3HK54			tr G3HK54	1	1	1	0.6	0.6	194.22	1768	1768	0	6.2436	0
47	P0AA16	P0AA16	Transcript ompR		sp P0AA16	1	1	1	7.9	7.9	27.353	239	239	0	6.2346	17463000
48	G3H0S3	G3H0S3			tr G3H0S3	1	1	1	0.8	0.8	154.93	1367	1367	0	6.1725	0
49	P0AEK2	P0AEK2	3-oxoacyl- fabG		sp P0AEK2	1	1	1	7	7	25.56	244	244	0	6.1606	7233600
50	P10378	P10378	Enterobac entE		sp P10378	1	1	1	2.1	2.1	59.111	536	536	0	6.1343	0
51	P23865	P23865	Tail-specif prc		sp P23865	1	1	1	1.5	1.5	76.662	682	682	0	6.1322	0
52	G3H5I4	G3H5I4			tr G3H5I4	1	1	1	1.6	1.6	42.241	381	381	0	6.0837	44038000
53	P24182	P24182	Biotin carb accC		sp P24182	1	1	1	3.3	3.3	49.32	449	449	0	6.0819	16330000
54	G3H4R2	G3H4R2			tr G3H4R2	1	1	1	0.7	0.7	187.86	1640	1640	1	-2	496750000
55	G3H8F6	G3H8F6			tr G3H8F6	1	1	1	2.7	2.7	44.742	409	409	1	-2	22738000
56	G3HEL8	G3HEL8			tr G3HEL8	1	1	1	12.9	12.9	8.0458	70	70	1	-2	544170000
57	G3HW82	G3HW82			tr G3HW82	1	1	1	0.6	0.6	120.71	1078	1078	1	-2	72127000
58	G3I4G3	G3I4G3			tr G3I4G3	1	1	1	4.7	4.7	27.252	233	233	1	-2	130670000
59	G3IMZ5	G3IMZ5			tr G3IMZ5	1	1	1	0.8	0.8	199.76	1736	1736	1	-2	9205000

b) Sample 2 (BPM Trial 2/20)

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Protein ID	Majority p	Fasta heac	Number o	Peptides	Unique pe	Sequence	Unique se	Mol. weig	Sequence	Sequence	Q-value	Score	Intensity
2	G3I386	G3I386	tr G3I386	1	1	1	1.1	1.1	78.965	704	704	0	323.31	121230000
3	G3HZE6;G	G3HZE6;G	tr G3HZE6	3	7	7	12.3	12.3	69.883	641	641;641;6	0	323.31	88068000
4	G3H9U3	G3H9U3	tr G3H9U3	1	4	3	15.1	12.8	40.238	351	351	0	85.195	126400000
5	G3HX10	G3HX10	tr G3HX10	1	1	1	2.3	2.3	33.53	303	303	0	43.985	4483200
6	G3HQP8;C	G3HQP8;C	tr G3HQP8	8	5	5	13.1	13.1	49.67	444	444;373;4	0	35.898	15736000
7	G3I7J6	G3I7J6	tr G3I7J6	1	4	3	6	4.7	69.615	613	613	1	-2	35810000

c) Sample 3 BPM Trial (3/3)

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	Protein ID	Majority p	Protein na	Gene nam	Fasta heac	Number o	Peptides	Unique pe	Sequence	Unique se	Mol. weig	Sequence le	Sequence	Q-value	Score	Intensity
2	G3I7J6	G3I7J6			tr G3I7J6	1	16	15	23	21.7	69.615	613	613	0	323.31	1392900000
3	G3H9U3	G3H9U3			tr G3H9U3	1	16	15	40.5	38.2	40.238	351	351	0	323.31	980540000
4	G3HZE6;G	G3HZE6;G3HZE5;G3HZE7			tr G3HZE6	5	8	7	18.7	16.7	69.883	641	641,641,6	0	323.31	342590000
5	P0A9K9	P0A9K9	FKBP-type slyD		sp P0A9K9	1	3	3	17.3	17.3	20.853	196	196	0	212.83	40232000
6	G3HG95	G3HG95			tr G3HG95	1	10	10	20.1	20.1	64.041	563	563	0	74.864	132550000
7	G3I386	G3I386			tr G3I386	1	1	1	1.1	1.1	78.965	704	704	0	38.422	210010000
8	G3HWN6	G3HWN6			tr G3HWN6	1	3	3	4.6	4.6	84.464	715	715	0	35.654	15900000
9	G3HHM5	G3HHM5			tr G3HHM5	1	1	1	0.1	0.1	760.28	6993	6993	0	34.28	5108700000
10	G3HQP8;C	G3HQP8			tr G3HQP8	6	5	5	20.5	20.5	49.67	444	444,373,4	0	33.21	55412000
11	G3HDG6;C	G3HDG6			tr G3HDG6	2	5	5	13.6	13.6	58.675	522	522,536	0	33.021	21936000
12	G3HHR3;C	G3HHR3			tr G3HHR3	2	4	4	9	9	53.742	466	466,471	0	26.236	34742000
13	G3HTU4	G3HTU4			tr G3HTU4	1	4	4	16.6	16.6	33.738	308	308	0	25.695	4726800
14	G3HII1	G3HII1			tr G3HII1	1	1	1	1.9	1.9	42.82	371	371	0	23.979	23772000
15	G3I6I6;G3	G3I6I6;G3INA2;G3HWE9;G3ICI3			tr G3I6I6	8	3	3	8	8	50.121	451	451,388,4	0	18.016	28755000
16	G3I6E6;G3	G3I6E6;G3GVD0;G3H5Q0;G3HC			tr G3I6E6	9	2	2	9.3	9.3	32.047	291	291,375,3	0	14.052	13613000
17	G3I3W6;G	G3I3W6;G3HP46;G3HWQ0;G3H			tr G3I3W6	5	2	2	14.9	14.9	16.577	148	148,131,1	0	12.706	15794000
18	G3HDU7;C	G3HDU7;G3HDT9;G3H2T6;G3H			tr G3HDU7	6	2	2	22.4	22.4	10.83	98	98,103,10	0	12.638	8861100
19	G3HX10	G3HX10			tr G3HX10	1	1	1	2.3	2.3	33.53	303	303	0	12.14	13011000
20	G3H180	G3H180			tr G3H180	1	1	1	0.4	0.4	254.48	2318	2318	0	11.627	172030000
21	G3GSG6	G3GSG6			tr G3GSG6	1	1	1	2.8	2.8	36.921	320	320	0	10.733	0
22	G3INX0;G	G3INX0;G3IB92;G3HPV1;G3HUY			tr G3INX0	10	1	1	11.9	11.9	13.934	126	126,126,1	0	8.9623	2819600
23	G3H2U6	G3H2U6			tr G3H2U6	1	1	1	3.3	3.3	44.355	424	424	0	8.6088	9224000
24	G3HTC0	G3HTC0			tr G3HTC0	1	1	1	1.2	1.2	96.121	837	837	0	8.4775	0
25	G3HRG8	G3HRG8			tr G3HRG8	1	1	1	5.4	5.4	29.729	258	258	0	7.5616	4334500
26	G3IJJ2;G3	G3IJJ2;G3ICG7;G3HED5			tr G3IJJ2	3	1	1	4.4	4.4	18.978	159	159,226,5	0	7.4803	11445000
27	G3GTC6	G3GTC6			tr G3GTC6	1	1	1	1.9	1.9	47.094	421	421	0	7.4527	2887000000
28	G3HMY4	G3HMY4			tr G3HMY4	1	1	1	1.9	1.9	41.376	370	370	0	7.2795	0
29	P17169	P17169	Glutamine glmS		sp P17169	1	1	1	3.4	3.4	66.894	609	609	0	6.7698	0
30	G3GSJ8	G3GSJ8			tr G3GSJ8	1	1	1	1	1	169.19	1536	1536	0	6.5413	0
31	G3HW82	G3HW82			tr G3HW82	1	1	1	0.6	0.6	120.71	1078	1078	0	6.5358	39212000
32	G3HTG1	G3HTG1			tr G3HTG1	1	1	1	1.5	1.5	45.854	413	413	0	6.4076	8141500
33	G3HRS2	G3HRS2			tr G3HRS2	1	1	1	3	3	22.977	201	201	0	6.3478	0
34	P64588	P64588	Transcript yqjI		sp P64588	1	1	1	3.4	3.4	23.401	207	207	0	6.2729	0
35	G3HJB6	G3HJB6			tr G3HJB6	1	1	1	2.5	2.5	71.794	651	651	0	6.2401	7792600
36	G3HTJ0;R	G3HTJ0;REV__G3HQB8			tr G3HTJ0	2	1	1	4.9	4.9	13.86	123	123,522	0	6.203	12766000
37	G3GR82	G3GR82			tr G3GR82	1	1	1	1.6	1.6	87.273	765	765	0	6.1691	414000000
38	G3H4G0	G3H4G0			tr G3H4G0	1	1	1	4.8	4.8	16.123	146	146	0	6.1686	9356900000
39	G3H220	G3H220			tr G3H220	1	1	1	2.4	2.4	49.363	423	423	0	6.1378	2196800
40	G3GZQ3	G3GZQ3			tr G3GZQ3	1	1	1	0.9	0.9	140.95	1254	1254	0	6.1187	88542000
41	G3HLL7;G	G3HLL7;G3IIF5;G3IIO7;G3IH98			tr G3HLL7	4	1	1	2.6	2.6	49.075	427	427,431,4	0	6.0924	6532500
42	G3HX84	G3HX84			tr G3HX84	1	1	1	1.8	1.8	72.077	658	658	0	6.091	49047000
43	G3IDL7	G3IDL7			tr G3IDL7	1	2	1	12	5.7	22.695	209	209	0	6.0844	1358200
44	G3GKK7	G3GKK7			tr G3GKK7	1	1	1	0.9	0.9	71.337	672	672	0	6.0143	160800000
45	G3IG70	G3IG70			tr G3IG70	1	1	1	1	1	91.472	771	771	0	5.9894	6666300
46	G3IM82	G3IM82			tr G3IM82	1	1	1	8.8	8.8	18.593	171	171	0	5.9857	33207000
47	G3HVJ5	G3HVJ5			tr G3HVJ5	1	1	1	1.7	1.7	92.069	803	803	0	5.9787	0
48	G3IIA9;G3	G3IIA9;G3IHL7			tr G3IIA9	2	1	1	3.9	3.9	37.501	333	333,333	0	5.9647	38816000
49	G3HGT8	G3HGT8			tr G3HGT8	1	1	1	5.8	5.8	14.501	121	121	0	5.9574	1981600
50	G3I301	G3I301			tr G3I301	1	1	1	0.4	0.4	559.43	5152	5152	0	5.9564	0
51	G3IL75	G3IL75			tr G3IL75	1	1	1	1.5	1.5	45.359	467	467	0	5.9304	3321600

d) Sample 4 (SrtA Control 1/30)

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	Protein ID	Majority p	Protein na	Gene nam	Fasta head	Number o	Peptides	Unique pe	Sequence	Unique se	Mol. weig	Sequence le	Sequence	Q-value	Score	Intensity
2	G3HZE6;G	G3HZE6;G3HZE5;G3HZE7			tr G3HZE4	8	10	10	17.8	17.8	69.883	641	641;641;6	0	323.31	362800000
3	P0A9K9	P0A9K9	FKBP-type slyD		sp P0A9K1	1	3	3	28.6	28.6	20.853	196	196	0	323.31	367420000
4	G3H9U3	G3H9U3			tr G3H9U1	1	7	7	23.6	23.6	40.238	351	351	0	88.979	454850000
5	G3I7J6	G3I7J6			tr G3I7J6	1	5	5	11.1	11.1	69.615	613	613	0	82.691	82221000
6	G3IB06;RE	G3IB06;REV__G3GRW5			tr G3IB06	2	2	2	11.8	11.8	8.1435	68	68;1195	0	59.43	1194900000
7	G3IKI6;G3	G3IKI6;G3IBP3			tr G3IKI6	2	2	2	4.2	4.2	92.388	835	835;441	0	58.914	81332000
8	G3I8F9;G3	G3I8F9;G3IPB6;G3IPJ0;G3IPN2;G3IPN3			tr G3I8F9	15	2	2	3.3	3.3	52.644	483	483;174;2	0	32.161	40692000
9	G3HX10	G3HX10			tr G3HX10	1	1	1	2.3	2.3	33.53	303	303	0	29.614	33089000
10	G3HJ86	G3HJ86			tr G3HJ86	1	2	2	4.6	4.6	71.794	651	651	0	13.314	42298000
11	G3IHJ2;G3	G3IHJ2;G3ICG7;G3HED5			tr G3IHJ2	3	1	1	4.4	4.4	18.978	159	159;226;5	0	9.5032	17798000
12	G3HL18	G3HL18			tr G3HL18	1	1	1	7.9	7.9	26.732	252	252	0	9.1317	47790000
13	G3H427	G3H427			tr G3H427	1	1	1	6.9	6.9	19.536	174	174	0	8.0312	8295100
14	G3HQQ7	G3HQQ7			tr G3HQQ7	1	1	1	2	2	102.25	959	959	0	7.4612	7052300
15	G3HLL7;G	G3HLL7;G3IIF5;G3IIO7;G3IH98			tr G3HLL7	4	1	1	2.6	2.6	49.075	427	427;431;4	0	7.3309	19423000
16	G3HWE1	G3HWE1			tr G3HWE1	1	1	1	2.4	2.4	52.033	454	454	0	6.9951	52980000
17	G3HAC6	G3HAC6			tr G3HAC6	1	1	1	0	0	4007	36026	36026	0	6.776	13359000
18	G3GTC6	G3GTC6			tr G3GTC6	1	1	1	1.9	1.9	47.094	421	421	0	6.5218	6525400000
19	G3HEX9	G3HEX9			tr G3HEX9	1	1	1	2.3	2.3	56.673	488	488	0	6.4617	285590000
20	G3IGI5	G3IGI5			tr G3IGI5	1	1	1	1.2	1.2	73.016	647	647	0	6.3917	697500000
21	G3H180	G3H180			tr G3H180	1	1	1	0.4	0.4	254.48	2318	2318	0	6.3526	834120000
22	G3IG44;G	G3IG44;G3HQP8;G3HCL2;G3GR			tr G3IG44	4	1	1	3.2	3.2	41.875	373	373;444;4	0	6.2768	3412000
23	G3HX84	G3HX84			tr G3HX84	1	1	1	1.8	1.8	72.077	658	658	0	6.2695	184500000
24	G3I2T9	G3I2T9			tr G3I2T9	1	1	1	1	1	89.604	799	799	0	6.0698	74901000
25	P76542	P76542	Putative p intZ		sp P76542	1	1	1	2	2	45.381	402	402	0	6.0698	16645000
26	G3GX63	G3GX63			tr G3GX63	1	1	1	49.3	49.3	7.1791	69	69	0	6.0661	3976000
27	G3GU88	G3GU88			tr G3GU88	1	1	1	1.3	1.3	65.307	547	547	0	6.0067	999350000
28	G3GV55	G3GV55			tr G3GV55	1	1	1	3.8	3.8	59.043	533	533	0	5.9878	0
29	G3H949	G3H949			tr G3H949	1	1	1	3.6	3.6	62.893	557	557	0	5.9878	0
30	P17888	P17888	Primosom priA		sp P17888	1	1	1	2.7	2.7	81.654	732	732	0	5.9878	0
31	G3HXS3	G3HXS3			tr G3HXS3	1	1	1	2.1	2.1	39.488	335	335	0	5.9337	94720000
32	G3GSR3	G3GSR3			tr G3GSR3	1	1	1	7.1	7.1	30.754	281	281	0	5.91	1888700
33	G3GZG6	G3GZG6			tr G3GZG6	1	1	1	0.9	0.9	134.6	1227	1227	0	5.8911	2707100

Figure 11. Mass spectrometry results from MtoZ Biolabs. List of proteins present in sample solutions and identified through proteomics database comparison.